의학석사 학위논문

Cloning and characterization of a recombinant gene for generation of CAR-iTreg harnessing CD40-CD154 interaction

CD40-CD154 상호작용을 이용한 CAR-iTreg 제작용 재조합 유전자의 클로닝

2019 년 07 월

서울대학교 대학원

의과학과 의과학전공

원 혜 지

CD40-CD154 상호작용을 이용한 CAR-iTreg 제작용 재조합 유전자의 클로닝

Cloning and characterization of a recombinant gene for generation of CAR-iTreg harnessing CD40-CD154 interaction

July 2019

The Department of Biomedical Science,
Seoul National University
College of Medicine
Hye Ji Won

ABSTRACT

Introduction: The regulatory T cells (Treg) are thymus-derived suppressor cells which play a critical role in maintaining immunological tolerance to self-antigens. Here, I describe a new approach to generating potent specific Tregs, using a chimeric antigen receptor (CAR) targeting CD40L. CARs are fusion proteins composed of an extracellular portion that is usually derived from antibodies and intracellular signaling portion derived from T cell signaling proteins. I generated a recombinant gene encoding a full length of the cytoplasmic domain of CD28, IL-2Rβ, TGF-βR1, and CD3ζ fused with a scFv of the MR1 mAb specific to CD40L as extracellular domain. Functionality of the cloned CAR was confirmed in EL4 cells by stimulation with HEK293 cells expressing CD40L.

Methods: Mouse $CD4^+$ T cell was isolated by magnetic-activated cell sorting (MACS) separation system according to the manufacturer's instructions. And this gene was used to compose the CAR construct. Each gene was obtained and extended using specific oligonucleotides by PCR. The synthesized CAR construct was inserted into a lentiviral expression vector. Using this vector and packaging vectors, the CAR was transfected and resulted in the production of lentivirus. The harvested virus was transduced in EL4 cell.

Results: The CAR construct was obtained and elongated from mouse cDNA. And, synthesized CAR constructs cloned into a lentiviral expression vector. The

expression of CAR was confirmed by ZsGreen1 fluorescence in pLVX-IRES-ZsGreen1 vector. Also, the cell line expressing CD40L of antigen to CAR was generated and confirmed by fluorescence of mCherry. The co-culture of EL4 cell expressing CAR on the response of HEK293FT cell expressing CD40L was conducted. The activation of EL4 cells was examined by CD69 expression level.

Conclusions: I describe a unique strategy to generate CAR. This novel CAR retains an identical costimulatory and CD3ζ signaling regions but also include an intracellular domain of IL-2Rβ and TGF-βR1. The inclusion of the intracellular domain of IL2-Rβ and TGF-βR1 enabled conversion of naïve T cells to iTreg in an antigen-dependent manner. Therefore, I expect this CAR construct would generate significant suppression effect in transplantation or autoimmune disease model. This CAR with CD40L specificity might provide a novel strategy to achieve tolerance in various immunopathologic conditions.

Keywords: Treg, CAR, PCR, Cloning, Transfection, Transduction.
Student number: 2017-22413

CONTENTS

LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS

Treg : Regulatory T cell

iTreg : inducible Treg

nTreg : natural Treg

CAR : Chimeric Antigen Receptor

scFv : single-chain variable fragment

MHC : Major Histocompatibility Complex

HLA : Human leukocyte antigen

APC : antigen-presenting cell

CD : Custer of Designation

MACS : Magnetic-Activated Cell Sorting

FACS : Fluorescence Activated Cell Sorting

PBS : Phosphate-Buffered Saline

MOG : myelin-oligodendrocyte glycoprotein

IL : Interleukin

TGF-β : Transforming Growth Factor-beta

STAT5 : Signal transducer and activator of transcription 5

PCR : Polymerase Chain Reaction

OE-PCR : Over extension Polymerase Chain Reaction

CD40L : CD40 ligand

pDNA : plasmid Deoxyribo Nucleic Acid

INTRODUCTION

The Tregs are thymus-derived suppressor cells and a subpopulation of T cells that modulate the immune system, maintain tolerance to self-antigens and prevent autoimmune disease. Tregs can be defined as a T cell population that functionally suppresses an immune response by influencing the activity of another cell type. So far, many studies have been done using Treg. Among them, the adoptive transfer is one of the methods for the treatment as cell therapy (1). Here, I describe what I believe is a new approach to generating potent specific Tregs, using a CAR targeting CD40L. I discussed here the generation and signaling properties of CARs, focusing on their conversion on T cell specificity and potency.

CARs are fusion proteins composed of an extracellular portion that is usually derived from antibodies and intracellular signaling portion derived from T cell signaling proteins (2). The feature of CAR is their ability to bind specific antigen with MHC independent manner and capable of directing the specificity of T cells toward desired antigens. I aimed to generate a new CAR-iTreg specific for CD40L. The structure of CAR molecule includes an scFv, a spacer, transmembrane domain, and an intracellular signaling domain. I generated lentiviral vector encoding a CAR specific for CD40L by cloning the scFv of the MR1 mAb and fusing the portions of CD28, IL-2Rβ, TGF-βR1, CD3ζ in a second generation CAR structure (Fig 1 A).

CAR-T cells are classified into three generations according to the existence of the costimulatory domain in the intracellular domain (3). The first generation

of CAR-T cells only has a single structure of CD3ζ in the intracellular domain. The CAR-T cells with CD3ζ were able to transmit signal like TCR into the cell. This first generation CAR-T cells were effective at killing tumor cells. But, these CAR-T cells did not get enough results because of insufficient proliferation, a short life span, and low expression of cytokine. Second generation CAR-T cells include the costimulatory molecule in the intracellular domain. CD28 is an essential mediator of T cells in the regulation of proliferation and survival (4). The second generation CAR-T cells transmit the costimulatory signal, such as CD28, 4-1BB or OX40, which can enhance the proliferation, cytotoxicity, life span of CAR-T cells. These CAR-T cells were conferred greater strength of signaling and persistence to the T cells, resulting in their overall higher potency. Third generation CAR-T cells have two costimulatory domain with CD3ζ in the intracellular domain. This CAR structure has improved proliferation and survival rate, but the killing ability was not improved compared to the second generation. So I synthesized the CAR based on second-generation design.

CAR-T cell is a promising and novel therapy for a variety of cancer models (5). This CAR-T cells with specificity to CD19 have proved the effective function of killing tumor cell as well as 70~90% complete response (6). So, CAR-T cell therapies were also studied for the treatment of a solid tumor. These CAR-T cell therapies have therapeutic potential, efficiency, and specificity (7). This therapy using CAR was being studied not only in the cancer model but also in autoimmune diseases. In autoimmune diseases that require immunosuppressive function, the use of CAR construct has been developed

using Treg. The CAR-Treg has been studied in colitis, which targets TNP, and in multiple sclerosis, which targets myelin-oligodendrocyte glycoprotein (MOG) (8). Unlike in autoimmune diseases, however, the study has yet to be done on the transplantation model.

It is a new and possible strategy to redirect the CAR with antigen specificity and applied it to the transplantation model. Tregs are immunosuppressive T cell with an important role in the maintenance of tolerance (9). In transplantation model, where the immune response takes place, Tregs that develop in the response of antigen presented by APC expand and proliferate. At first, the polyclonal Treg were used to suppress the immune response. Polyclonal Treg cells can suppress effector T cells with various specificity, but it is not enough to suppress the reject response after transplantation of graft because of the lack of donor antigen-specific Treg (10). So, Tregs have evolved with specificity to donor antigen by donor APC. Alloantigen-specific Tregs are more effective than polyclonal Tregs. But, as a therapy, there were limits to need for donor APC and long expansion time. So, next step, the CAR-Treg have been engineered with antigen specificity, applied to the transplanted mouse, and accepted graft *in vivo* (11).

Graft-specific CAR-Tregs changing nTregs via the transfer of CAR designed to recognize the MHC class allele A*02 was generated (12). These A2-CAR Tregs exhibited higher proliferative capacity, stable phenotype, and suppressing function than nTregs. Also, HLA-A2 specific CARs were engineered. These redirected CAR-Treg toward MHC-class I molecule represented superior to protect the graft from allograft rejection *in vitro* and a

human skin xenograft transplant model (13). These results demonstrated the possibility of CAR technology for clinical trials and new potential therapy. Thus, the CAR-Treg has an exceptionally positive result as a therapy, but there is a limit of separating the T reg and transducing the CAR. So, I designed that the naïve T cell could be conversed into Tregs by antigen stimulation, using CAR technology for more powerful suppressive function and proliferative capacity.

Naïve $CD4^{+}CD25^{-}$T cells could be conversed into Tregs by co-stimulation with T cell receptors (TCRs) and transforming growth factor-β (TGF-β) (14). To induce TGF-β activation in CAR T cells in an antigen-dependent manner, I include a full length of the cytoplasmic domain of the TGF-β receptor in the intracellular domain. TGF-β receptors have three types, among them, TGF-βR3 involved in capturing antigen, TGF-βR2 forms a complex with TGF-βR1 and then activated TGF-βR1 phosphorylates SMAD2 (15). It is a critical role in the induction of TGF-β signaling is TGF-βR1-mediated. TGF-β and IL-6 induce naïve T cells to become a Th17 cell. Moreover, IL-6 can convert Treg to Th17 cells. IL-2 and TGF-β stimulation lower IL-6 receptor expression and IL-6 signaling, resulting in the blocking of Tregs to Th17 conversion (16). So I included a full length of the signaling domain of the IL-2 receptor in the intracellular domain next to TGF-β receptor. IL-2 receptors have three types, among them, IL-2Rα involved in increasing the antigen binding affinity, IL-2Rβ and IL-2Rγ formed dimerization and induce signal transduction (17). Primarily, IL-2R β provide the docking site of JAK and results in phosphorylation of STAT5.

During the generation of the immune response, several molecular signals are needed. A first signal is the engagement of the antigen receptor to antigen by T cell and APC. The second signal is the binding of costimulatory molecule expressed by T cell and APC with their ligands. CD40 bind CD40L, which is expressed on activated T cell results in modulating immune responses (18). So, the block of CD40/CD40L engagement might play an essential role in transplantation condition. Anti-CD40L co-stimulation blockade treated mice accepted a second graft without graft loss (19). It shows a possibility to study the mechanism of tolerance and new replacing therapy. But, there are limit anti-CD40L blockade induces platelet activation and thrombosis results in critical issue in transplantation condition as well as an autoimmune disease model (20). Then, the research exhibited that an anti-CD40L blockade lacking Fc domain prevent thrombosis with successful immune suppressive function (21). Also, glycosylated anti-CD40L blockade still has a useful effect of preventing graft loss after transplantation (22). Therefore, the use of anti-CD40L support useful target therapy for transplantation and autoimmune disease. So, I determined the specificity of CAR to CD40L.

MATERIALS AND METHODS

1. Isolation of Mouse $CD4^+$ T cell

Splenic $CD4^+$T cell was isolated from mice by MACS magnetic separation system according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, spleen cell suspensions were incubated in cold PBS buffer with biotin-antibody cocktail/anti-biotin microbeads according to the manufacturer's instructions ($CD4^+$ T cell isolation kit, Miltenyi Biotec). The LS columns were placed in the magnetic field of MACS separator and rinsed. The suspension cell was loaded onto the LS columns result in $CD4^+$ T cell were collected.

2. PCR/Over-Extension PCR (OE-PCR)

The OE-PCR consists of two DNA of interest and two oligonucleotide primer, which generate single-stranded DNA that is annealed together and extended under appropriate condition. The OE-PCR-based synthesis of the gene involved two rounds of PCR. The first rounds are to extend the gene fragment (*Cd3ζ*, *Cd28*) of approximately 20 bp corresponding to be connected to the next. Two pairs of an overlapping fragment from the first round were extended the one fragment in the second rounds PCR using the corresponding two-end primer of the connected gene. The PCRs were conducted as follows: a 5 min initial denaturation at 95°C; 35 cycles of 94°C for 30 s, annealing step (depends on primer condition), and 72°C for 30 s; and a final step of 5 min at 72°C to ensure complete extension.

3. Oligonucleotides

The oligonucleotide was purchased from COSMO-genetech (Seoul, Korea) and mBiotech (Gyeonggi-do, Korea). The oligonucleotides were diluted to 10 mM with 3 distilled water (DW) before use.

4. Cell line

Both HEK293FT and EL4 cell line are obtained from American Type Culture Collection (ATCC, Manassas, VA). These cells were grown in DMEM-based media (Life Technologies, Carlsbad, CA). The culture media were supplemented with 10% heat-inactivated FBS, MEM-NEAA, HEPES, 2-Mercaptoethanol, gluta-MAX (Life Technologies). All cells were grown at 37°C in the presence of 5% CO_2.

5. Gel Electrophoresis/Sequencing

Separation of DNA in 1% (±0.5) agarose gel were run at 100 V for 20 m in TAE buffer. The gel was exposed to UV light and the picture taken with a gel documentation system. Premix with Taq polymerase (Bioneer, Chungcheong-do, Korea) and premix with Pfu polymerase (Mgmed, Seoul, Korea) were used during PCR. The 10 μl of the amplificated PCR product were loaded to each lane. The 4 μl of Gel marker from COSMO gentech were loaded each lane. The synthesized gene fragments were obtained by 1% (±0.5) agarose gel using electrophoresis and were purified by PCR purification kit (COSMO-genetech)

or Gel extraction kit (COSMO-genetech). The elution was performed with 40 μl. The DNA sequences were analyzed by COSMO-genetech.

6. Transfection

HEK293FT cells were co-transfected with plVX-IRES-ZsGreen1 (TAKARA Bio, Seoul. Korea) with CAR, pLP1 (Invitrogen, Carlsbad, CA), pLP2 (Invitrogen), PLP/VSVG (Invitrogen) pDNA at a mass ratio of 1:1:1:1 using fugene HD transfection reagent. pDNA was prepared by Mini prep (Qiagen, Hilden, Germany), according to the manufacturer's instructions. A total of 15 μg of pDNA was added to 5×10^6 HEK293FT cells in 8 ml Opti-MEM media (Life Technologies). The media was changed at 24 h post-transfection. Viral supernatant was harvested 72 h post-transfection.

7. Transduction of T cells

The retrovirus with CAR-expressing pDNA was used for the infection of the EL4 cell line for 48 h. The transduction was performed on days 2 following the stimulation with anti-CD3/CD28 (eBioscience Inc, San Diego, CA) at a concentration of 5 μg/ml. And the expression was checked on day 4 or 5.

8. FACS

HEK293FT cell and EL4 cell were washed twice for 5 min in 1 ml FACS buffer at 500 x G. At the end of the washing step, the pellet was resuspended in 200 μl FACS buffer and analyzed using a FACS Canto flow cytometer (BD

Bioscience, Franklin Lakes, NJ) The data were analyzed using flowjo software (BD Bioscience)

RESULTS

Generation of the intracellular domain of the CAR

The full length of mouse *Cd28* sequence except for stop codon was PCR cloned with 5' oligonucleotide forward primer CD28F (including Spe I site) and 3' oligonucleotide reverse primer CD28R (including Spe I site), consisting of nucleotide 97-750 (Fig 1 B and 2 A, Table 1). The cytoplasmic sequence for CAR contains nucleotide 954-1766 of mouse *Il2rβ*. This gene was PCR cloned with 5' oligonucleotide forward primer IL2RβBF (including a Spe I site) and 3' oligonucleotide reverse primer IL2RβR (including an Hinc II site). The stop codon of *Il2rβ* gene was eliminated (Fig 1 B and 2 B, Table 1). The cytoplasmic sequence for CAR contains nucleotide 532-1599 of mouse *Tgfβr1* excepting stop codon. This gene was PCR cloned with 5' oligonucleotide forward primer TGFβR1F (including a Spe I site) and 3' oligonucleotide reverse primer TGFβR1R (including an Hinc II site) (Fig 1 B and 2 C, Table 1). The signaling domain of cytoplasmic sequence for CAR contains nucleotide 302-643 of mouse *Cd3ζ*. This gene was PCR cloned with 5' oligonucleotide forward primer CD3ζF (including an Hinc II site) and 3' oligonucleotide reverse primer CD3ζR (including a Not I site) (Fig 1 B and 2 D, Table 1). This PCR reaction produced single bands, which matched the expected size. Each primer sequence with specific restriction endonuclease site is incorporated into the PCR product during amplification. The single bands were obtained and modified by TA cloning method and resulted in the production of the form of plasmid DNA.

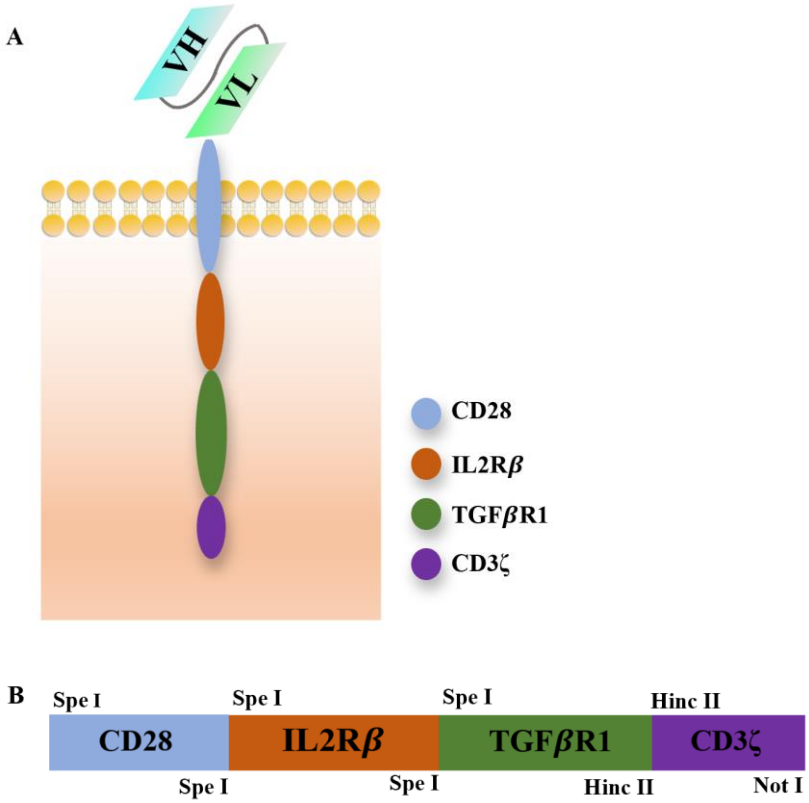

Figure 1. Schematic diagram describing the component of the CAR genes.

A) Scheme of CAR containing scFvs, CD28 hinge region, CD28 transmembrane domain, CD28 costimulatory domain, IL2Rβ intracellular domain, TGFβR1 intracellular domain, and CD3ζ.

B) Diagram of the restriction endonuclease site.

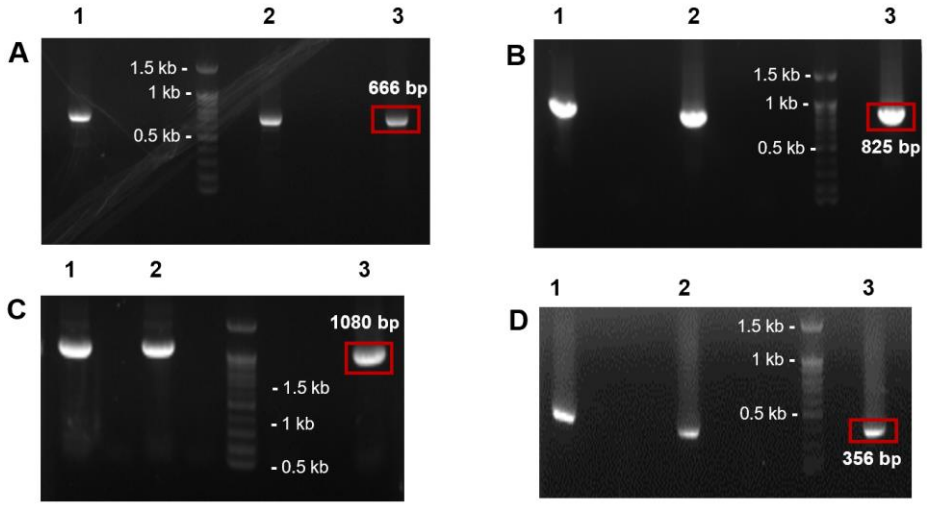

Figure 2. Acquisition of cytoplasmic gene.

A PCR fragment from mouse cDNA was amplified by different annealing temperature as summarized in Table 1. Lane 3 was extracted. A) A CD28 PCR fragment of 666 bp from mouse cDNA. B) A IL2Rβ PCR fragment of 825 bp from mouse cDNA. C) A TGFRβR1 PCR fragment of 1080 bp from mouse cDNA. D) A CD3ζ PCR fragment of 356 bp from mouse cDNA.

Gene	Primer name	Lane	Annealing temperature
CD28	CD28F **ACTAGT** ATGACACTCAGGCTGCTG	1	60.9℃
		2	62.3℃
	CD28R **ACTAGT** GGGGCGGTACGCTGCAAAG	3	63.8℃
IL2Rβ	IL2RβF **ACTAGT** AAGTGCCGGTACCTTGGG	1	59.6℃
		2	60.2℃
	IL2RβR **ACTAGT** TATTAGGTGGACGAATC	3	61.6℃
TGFRβR1	TGFRβR1F **ACTAGT** TGCCATAACCGCACTGTC	1	60.9℃
		2	62.3℃
	TGFRβR1R **GTCGAC** CATTTTGATGCCTTCCTG	3	63.8℃
CD3ζ	CD3ζF **GTCGAC** GAGCAAAATTCAGCAGGAC	1	61.6℃
		2	62.3℃
	CD3ζR **GCGGCCGC** TTAGGGAGGGGCCAG	3	63.1℃

Table 1. The PCR conditions for acquisition of the cytoplasmic domain of CAR.

The PCR product was amplified with specific restriction enzyme site using these oligonucleotide primers. The bold lettering indicates restriction endonuclease sites.

To induce conversion of CAR-T cell to inducible CAR-regulatory T cells (CAR-iTreg) in an antigen-dependent manner, I incorporated a full length of the cytoplasmic domain of *Il2rβ* and *Tgfβr1* between the cytoplasmic domain of *Cd28* and *Cd3ζ*. To generate synthesized construct of the cytoplasmic domain, I elongated *Cd3ζ* and *Cd28* pDNA using CD3ζ(oe)F/CD3ζR and CD28F/CD28(oe)R respectively (Fig 3 A and B, Table 1 and 2). This particular primer contains about 20 bp of the overlapping end to be connected and results in the generation of 5' or 3' overhang on PCR product that is complementary to sequences at the end of each gene to be connected (Table 2). So, *Cd3ζ* gene was extended by including *Tgfβr1* gene at 5' ends. And *Cd28* gene was extended by including *Il2rβ* gene at 3' ends. The OE-PCR using 'CD28F and IL2RβR primers' and 'extended *Cd28* gene fragment and *Il2rβ* gene fragment' as the template generated correct PCR product with expected DNA length. Likewise, The OE-PCR using 'TGFβR1F and CD3ζR primers' and '*Tgfβr1* gene fragment and extended *Cd3ζ* gene fragment' as the template generated correct PCR product with expected DNA length (Fig 3 C, Table 1). This PCR product was extracted and cloned to the T vector.

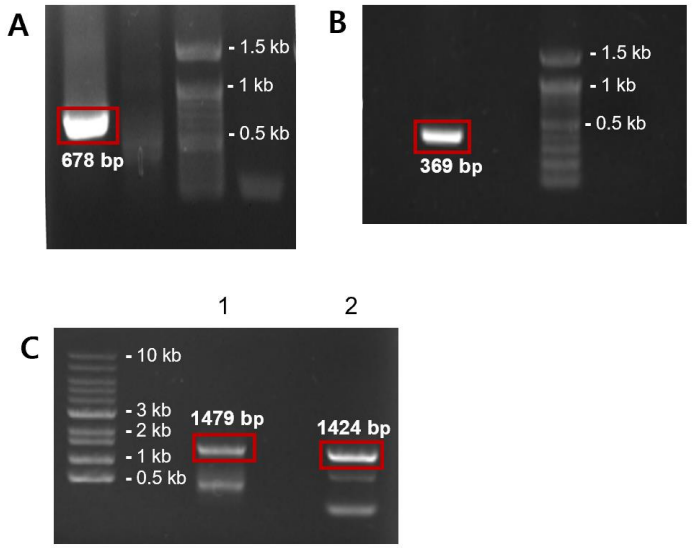

Figure 3. Elongation and synthesis of the gene for the cytoplasmic domain of CAR.

A) The elongated PCR product using CD28F/CD28(oe)R primer sets. The PCR product of Fig2 A was used as a template. B) The elongated PCR product using CD3ζ(oe)F/CD3ζR primer sets. The PCR product of Fig2 D gene was used as a template. C) The result of OE-PCR. Lane 1 and 2 represent a *'Cd28+Il2rβ'* and '*Tgfβr1+Cd3ζ'* gene fragment, respectively. The PCR product with the expected length of the gene was confirmed.

	IL2Rβ CD28
CD28(oe)R	CCCAAGGTACCGGCACTT GGGGCGGTACGCTGCAAAG
CD3ζ(oe)F	ACAGGAAGGCATCAAAATG AGAGCAAAATTCAGCAG
	TGFβR1 CD3ζ

Table 2. The unique oligonucleotide sequences for OE-PCR.

The CD28(oe)R primer containing 18 bp of 5' IL-2Rβ gene synthesized the templates containing 3' overhang of IL-2Rβ. The CD3ζ(oe)F primer containg 19 bp of 3' TGFβR1 gene synthesized the templates containing 5' overhang of TGFβR1.

Each pDNAs could cleaved with appropriate restriction endonuclease (Spe I). The cleavage reaction resulted in the formation of the linearized part of pDNA with the restriction endonuclease site at the end of this fragment. The T vector, including *Cd28+Il2rβ* gene was separated two strands as insert and T vector with Spe I restriction enzyme site at the end of strands. And the T vector, including *Tgfβr1+Cd3ζ* gene was cut and produced long linear sequence including *Tgfβr1+Cd3ζ* gene on T vector with Spe I restriction endonuclease site (Fig 4 A). The product of restriction endonuclease digestion, '*Cd28+Il2rβ*' fragment to be cloned into the sticky end of '*Tgfβr1+Cd3ζ'* containing T vector by T4 ligase, and transformed into competent *E coli*. The transformation products were screened using a plate with antibiotics for selection. The colonies were identified by PCR at the expected DNA size using universal M13F/M13R primers (Fig 4 B). Among the checked transformed product, the second PCR was performed using M13R/CD28R primers for checking the correct orientation (Fig 4 C, T able 1).

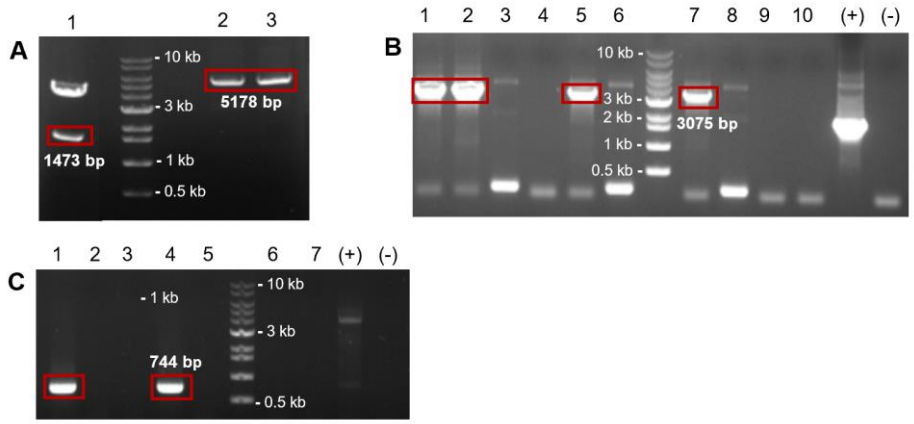

Figure 4. Generation of the signaling domain of CAR.

A) Separation of the Spe I digested pDNA. Lane 1 represents separated two strands (T vector, *Cd28+Il2rβ* from the top side). Lane 2 and 3 represent linearized T vector including '*Tgfβr1+Cd3ζ'* gene. The fragment estimated gene size 1473 bp (*Cd28+Il2rβ*) and 5178 bp (*Tgfβr1+Cd3ζ*) were extracted and purified. B) Screening of colonies for the presence of insertion. The PCR was implemented using M13R/M13F primer sets. The PCR fragment with 3075 bp was confirmed and extracted. The lane 1,2,5 and 7 represent a correct band size. C) Confirmation of the insert orientation. The PCR was conducted with M13R/CD28R primer sets. The PCR fragment with 744 bp length was confirmed and extracted. Lane 1 and 4 represent a correct band size.

Generation of the extracellular domain of the CAR.

The extracellular domain of CAR consists of the leader sequence, V region of the H chain of linked a $(G_4S)3$ linker to the V region of L chain of the MR1 Ab (23). DNA for single-chain fragment variable (scFv) was obtained from MR1 hybridoma to target CD40L (24). I synthesized V_H-F and V_H-R primer, which contained the part of leader sequence and 5' end of the V_H gene, and the 3' end of the V_H gene, respectively (Table 4). I amplified the V_H gene using V_H -F/ V_H-R sets from genomic DNA of MR1 cell line (Fig 6 A, Table 3). Likewise, I synthesized V_L-F and V_L-R primer, which contained the part of leader sequence and 5' end of the V_L gene, and the 3' end of the V_L gene respectively (Table 4). I amplified the V_L gene using V_L-F/ V_L-R sets from genomic DNA of MR1 cell line (Fig 6 B, Table 3).

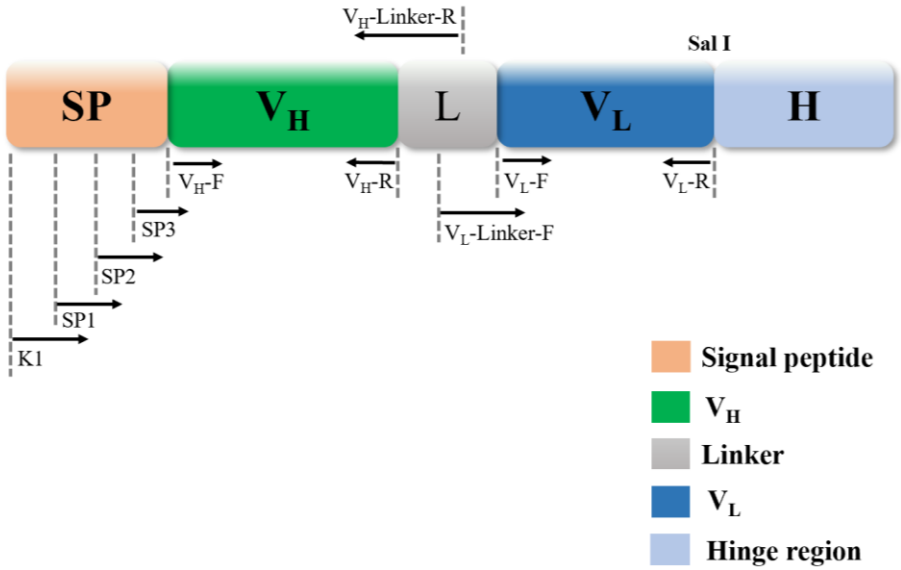

Figure 5. Schematic diagram presenting the extracellular domain of the CAR.

Scheme of the extracellular domain of CAR with oligonucleotides.

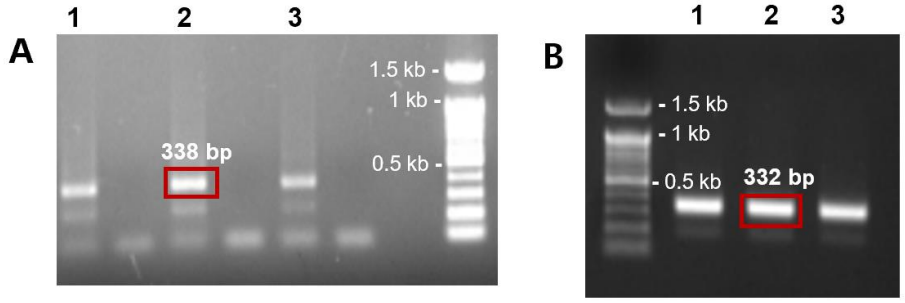

Figure 6. Acquisition of the scFv gene.

A PCR fragment from cDNA of MR1 hybrydoma was amplified by different annealing temperature as summarized in Table 3. Lane 2 was extracted. A) A V_H PCR fragment with 338 bp. B) A V_L PCR fragment with 332 bp.

Gene	Primer name	Lane	Annealing temperature
V_H	V_H-F	1	57.5℃
	V_H-R	2	59.5℃
		3	61.5℃
V_L	V_L-F	1	50.4℃
	V_L-R	2	53.4℃
		3	55.5℃

Table 3. The PCR conditions for acquisition of the scFv.

	Leader sequence ← V_H →
V_H-F	GTGTCCACTCC CAGGTCCAG
V_L-F	GTGCCTGTGCA GATATCGTG
	Leader sequence ← V_L →
V_H-R	TGAGGAGACGGTGACCATG
V_L-R	TTTTATCTCCAGCTTGGTGC

Table 4. The oligonucleotide sequences for the acquisition of scFv.

The V_H-F primer and V_L-F containing of 11 bp of 5' leader sequence synthesized the template containing 5' overhang of leader sequence.

The 30 nucleotides of linker sequence are synthesized as the part of V_H-linker-R primers and are added to the 3' end of the V_H gene during PCR with V_H-F primer using V_H gene as a template (Fig 7 A, Table 5 and 6). And, 33 nucleotides of linker sequence are synthesized as the part of V_L-linker-F primers and are added to the 5' end of the V_L gene during PCR with V_L-R primer using V_L gene as a template (Fig 7 B, Table 5 and 6). This special primer has repeated nucleotide encoding 'G_4S' amino acid sequences. So, the PCR product results in the extended DNA sequence for the linker sequences. The signal peptide of the CAR consists of nucleotide 1-81 of mouse CD8 alpha chain (25). Using SP3/ V_H-linker-R primers set, SP2/V_H-linker-R primers set and V_H-linker gene as a template, the signal peptide sequence was extended by PCR in sequential (Fig 7 C and D, Table 5 and 6). Using these special oligonucleotide, the part of leader sequence on V_H-F/ V_L-F primer sets were deleted.

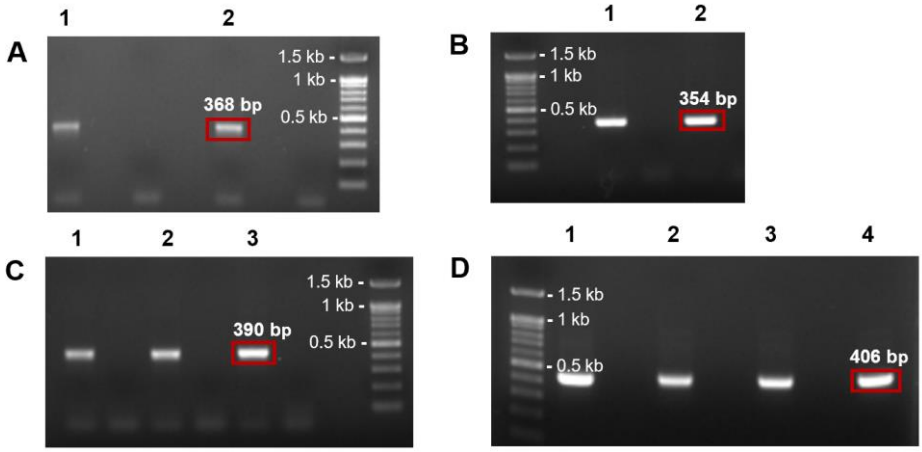

Figure 7. Elongation and synthesis of the gene for the extracellular domain of CAR.

The result of gradient PCR with different annealing temperature as summarized in Table 5. A) The elongated PCR product using V_H-F/V_H-Linker-R primer sets and PCR product of Fig6 A gene as a template. B) The elongated PCR product using V_L-Linker-F/V_L-R primer sets and PCR product of Fig6 B gene as a template. C) The elongated PCR product using SP3/V_H-Linker-R primer sets and PCR Product of Fig7 A gene as a template. D) The elongated PCR product using SP2/V_H-Linker-R primer sets and PCR product of Fig7 C gene as a template.

Gene	Primer name	Lane	Annealing temperature
V_H+linker	V_H-F	1	56℃
	V_H-Linker-R	2	58℃
V_L+linker	V_L-Linker-F	1	52℃
	V_L-R	2	59℃
Signal peptide + V_H+linker	SP3	1	52℃
	V_H-Linker-R	2	59℃
		3	65℃
Signal peptide + V_H+linker	SP2	1	52℃
	V_H-Linker-R	2	54.1℃
		3	60℃
		4	64℃

Table 5. The PCR conditions for acquisition of the extracellular domain of CAR.

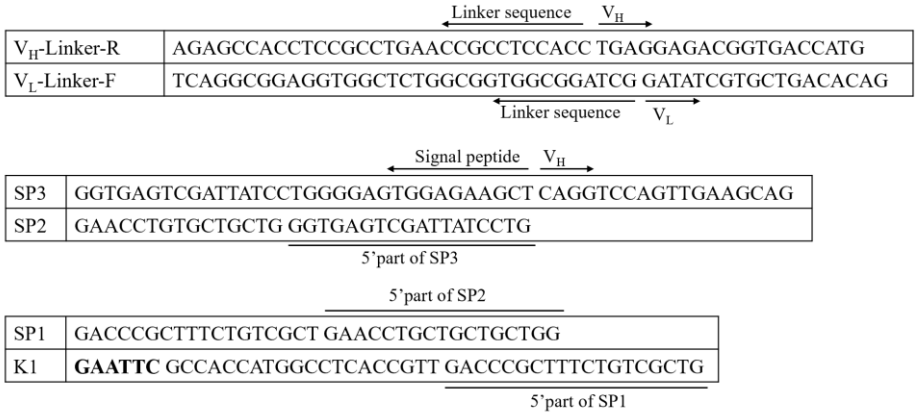

Table 6. The unique oligonucleotide sequences for extension of the scFv gene fragment.

The V_H-Linker-R primer containing of 30 bp of 5' linker sequence synthesized the template containing 3' overhang of linker sequence. The V_L-Linker-R primer containing of 33 bp of 3' linker sequence synthesized the template containing 5' overhang of linker sequence. The SP3 primer is complementary to 18 bp of 5' V_H and 33 bp of 3' signal peptide genes. The SP2 primer is complementary to 18 bp of 5' SP3 and 15 bp of middle part of signal peptide genes. The SP1 primer is complementary to 17 bp of 5' SP2 and 18 bp of middle part of signal peptide genes. The K1 primer is complementary to 19 bp of 5' SP1 and 20 bp 5' signal peptide genes with restriction endonuclease site. The bold lettering indicated restriction endonuclease sites.

The OE-PCR using SP2/V_L-R-Sal I primers set and 'SP2+V_H+Linker gene and Linker+V_L gene' as the template generated correct PCR product with expected DNA length (Fig 8 A, Table 6, 7 and 9). The V_L-R- Sal I primer has specific restriction enzyme site result in PCR product with Sal I sequence at the 3' end during amplification by PCR. The PCR product was extended using SP1/V_L-R-Sal I primer set and then K1/V_L-R-Sal I set by PCR in sequential (Fig 8 B and C. Table 6, 7 and 9). These PCR products were extracted and cloned to the T vector. The transformation products were screened using a plate with antibiotics for selection.

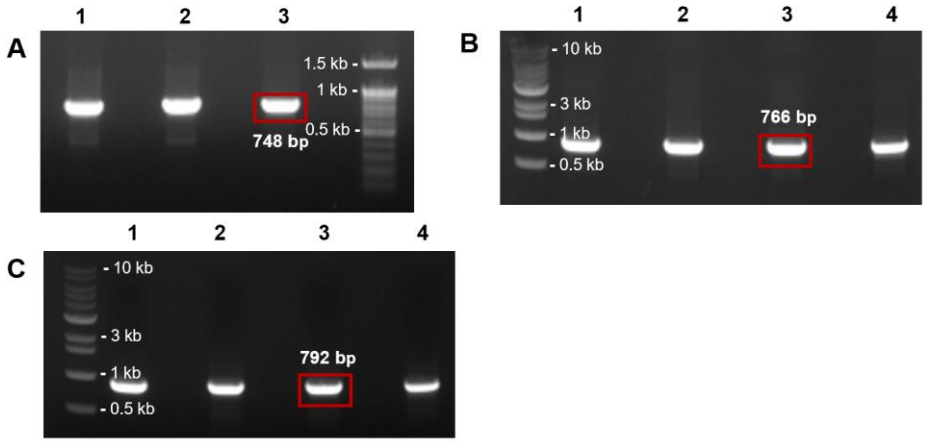

Figure 8. Generation of the extracellular domain of CAR.

The result of PCR with different annealing temperature as summarized in Table 7. A) The result of OE-PCR. The PCR was implemented using SP2/V_L-R-Sal I primer sets and PCR product of Fig7 B/Fig7 D gene as a template. B) The elongated PCR product using SP1/V_L-R-Sal I primer sets and PCR product of Fig8 A gene as a template. C) The elongated PCR product using K1/V_L-R-Sal I primer sets and PCR product of Fig8 B gene as a template.

Gene	Primer name	Lane	Annealing temperature
Signal peptide +V_H+Linker +V_L	SP2 V_L-R-Sal I	1	54.1℃
		2	58℃
		3	64℃
Signal peptide +V_H+Linker +V_L	SP1 V_L-R-Sal I	1	58℃
		2	62℃
		3	66℃
Signal peptide +V_H+Linker +V_L	SP1 V_L-R-Sal I	1	56℃
		2	58℃
		3	62℃
		4	66℃

Table 7. The PCR conditions for acquisition of the extracellular domain of CAR.

Synthesis of CAR structure.

The extracellular spacer region consists of residues 340-450 of mouse *Cd28* region (26). The intracellular domain of CAR with spacer region was generated using Hinge-CD28-Sal I/CD3ζ-Not I primer sets by PCR with pfu polymerase (Fig 9 A, Table 8 and 9). These PCR products were extracted and cloned to the T vector. The transformation products were screened using a plate with antibiotics for selection. The size of the insertions in the transformants using M13F/M13R primers sets has been confirmed by PCR. The pDNA of scFv and Ecto/cytoplasmic domain gene can be cleaved with appropriate restriction endonucleases (Sal I and Not I) (Fig 9 B). The cleavage reaction results in the formation of the linearized part of pDNA with the restriction endonuclease site at the end of this fragment, respectively. This product was cloned into the sticky end of 'scFv' vector by T4 ligase and transformed. The transformation products were screened using a plate with antibiotics for selection. The colonies were identified by PCR at the expected DNA size.

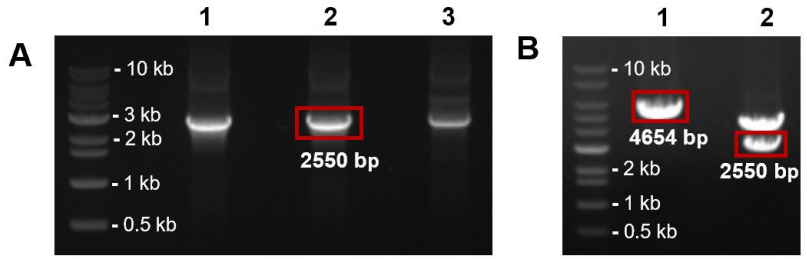

Figure 9 Generation of CAR on the T vector.

A) The PCR was implemented using Hinge-CD28-Sal I/CD3ζR primer sets and PCR product of Fig4 C gene as a template with different annealing temperature as summarized in Table 8. B) Separation of Sal I and Not I digested pDNA. Lane 1 represents linearized T vector including scFv gene. Lane 2 represent separated two strands (T vector, ecto/cytoplasmic domain from the top side). The fragment estimated gene size 4654 bp (scFv+T vector) and 2550 bp (ecto/cytoplasmic domain) from the left side were extracted and purified.

Gene	Primer name	Lane	Annealing temperature
CD28 +IL2Rβ +TGFβR1 +CD3ζ	Hinge-CD28-Sal I CD3ζR	1	52℃
		2	59℃
		3	65℃

Table 8. The PCR conditions for acquisition of the intracellular domain of CAR.

Hinge-CD28-Sal I	**GTCGAC** AAAATTGAGTTCATGTAC
V_L-R-Sal I	**GTCGAC** TTTTATCTCCAGCTTGGTGC

Table 9. The unique oligonucleotide sequences for production of gene fragment.

The bold lettering indicated restriction endonuclease sites**.**

Transfer of CAR structure to a lentiviral expression vector.

I attempted inserting the CAR DNA construct into pLVX-IRES-zsGreen1 vectors. With such a long sequence of the CAR, it was challenging to generate lentiviral expression vector with CAR as an insert. So, I have implemented the transfer of CAR structure to Lentivirus expression vector in two steps. First, The pDNAs were cleaved with appropriate restriction endonuclease (EcoR I, Spe I and Not I) (Fig 10 A). The cleavage reaction resulted in the formation of the linearized part of pDNA with the specific restriction endonuclease site at the end of this fragment, respectively. The pDNA of CAR on T vector was cut by restriction endonuclease (EcoR I, Spe I and Not I) result in 3 linearized strands (T vector, '*scFv~Il2rβ* and *Tgfβr1+Cd3ζ*). The '*scFv~Il2rβ*' fragment has an EcoR I restriction endonuclease site at the end of 5' and a Spe I restriction endonuclease site at the end of 3'. The '*Tgfβr1+Cd3ζ*' fragment has a Spe I restriction endonuclease site at the end of 5' and a Not I restriction endonuclease site at the end of 3'. And, the '*Tgfβr1+Cd3ζ*' fragment was cloned into pLVX-IRES-ZsGreen1 vector cleaved by restriction endonucleases (Spe I and Not I), and transformed. The transformation products were screened using a plate with antibiotics for selection. The colonies were identified by PCR at the estimated DNA size. Second, these '*Tgfβr1+Cd3ζ*' on Lentivirus expression vector fragment was cleaved by restriction endonuclease (EcoR I and Spe I). And, the cut '*scFv~Il2rβ* gene' was cloned into this vector and transformed. The transformation products were screened and checked at the estimated DNA size. The cleavage reaction was conducted to determine the DNA size in the lentiviral expression vector and the size was identified (Fig 10 B). Taken together, the sequence of CAR construct was confirmed and explained (Fig 11 and 12).

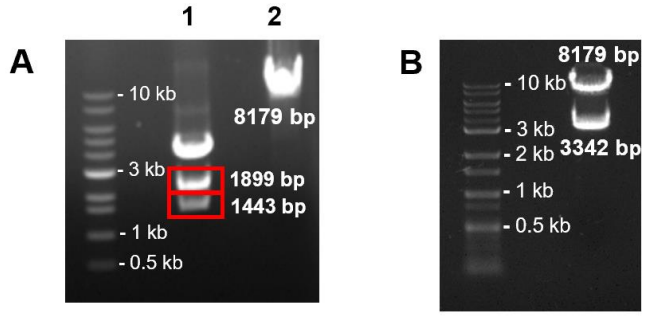

Figure 10. Generation of CAR on Lentiviral expression vector.

A) Separation of Spe I, Not I and EcoR I digested pDNA. Lane 1 represents the pDNA including CAR separated three strands (T vector, *scFv+Cd28+Il2rβ* gene and *Tgfβr1+Cd3ζ* gene from the top side). Lane 2 represent the linearized lentiviral expression vector. The bands with cut CAR gene were extracted and purified. B) Separation of EcoR I and Not I digested pDNA of a lentiviral expression vector including CAR.

pLVX-F	GCAGAGCTCGTTTAGTGAAC
pLVX-R	GCAATATGGTGGAAAATAAC

Table 10. The unique oligonucleotide sequences.

This primer sequence was synthesized based on pLVX-IRES-ZsGreen1 vector gene.

Figure 11. Nucleotide sequence of CAR construct.

		Nucleotide sequence
Extracellular domain	Signal peptide	GCCACCATGGCTCACCGTTGACCCGCTTTCTGTCGCTGAACCTGCTGCTGC TGGGTGAGTCGATTATCCTGGGGAGTGGAGAAGCT
	V_H	CAGGTCCAGTTGAAGCAGTCTGGGGCTGAGTTTGTGAAGCCTGGAGCCTC AGTGAAGATATCCTGCAAAACTTCAGGCTATACCTTCACTGATGGCTACAT GAACTGGGTTGAGCAGAAGCCTGGGCAGGGCCTTGAGTGGATTGGAAGAA TTGATCCTGATAGTGGTGATACTAGGTACAATCAGAAGTTCCAGGGCAAGG CCACACTGACTAGAGACAAATCCTCCAGCACAGTCTACATGGACCTCAGGA GTCTGACATCTGAGGACTCTGCTGTCTATTACTGTGCGAGAGCCCCTTATA TAGCGGATATAGGGGAGGCCTTTGATTACTGGGGCCAAGGAACCATGGTC ACCGTCTCCTCA
	Linker	GGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCG
	V_L	GATATCGTGCTGACACAGTCTCCATCTTCCTTGGCTGTGTCCGCAGGAGA CAAGGTCACCATCAACTGCAAGTCCAGTCAGAGTCTTTTATCTGGTGGCT ATAACTACTTGGCTTGGTACCAGCAGAAAACAGGGCAGTCTCCTAAATTA CTGATCTATTTCACATCCACTCGGCACACTGGTGTCCCTGATCGCTTCAT AGGCAGTGGGTCTGGGACAGATTTCACTCTAACCATCAACAGTTTCCAGA CTGAGGATCTGGGAGATTACTATTGTCAGCATCATTACGGTACTCCTCTC ACGTTCGGTGATGGCACCAAGCTGGAGATAAAA
Transmembrane domain	CD28	AAAATTGAGTTCATGTACCCTCCGCCTTACCTAGACAACGAGAGGAGCAA TGGAACTATTATTCACATAAAAGAGAAACATCTTTGTCATACTCAGTCATC TCCTAAGCTG
		TTTTGGGCACTGGTCGTGGTTGCTGGAGTCCTGTTTTGTTATGGCTTGCT AGTGACAGTGGCTCTTTGTGTTATCTGGACA
Intracellular domain		AATAGTAGAAGGAACAGACTCCTTCAAAGTGACTACATGAACATGACTCC CCGGAGGCCTGGGCTCACTCGAAAGCCTTACCAGCCCTACGCCCCTGCCA GAGACTTTGCAGCGTACCGCCCC
	IL2R β	AAGTGCCGGTACCTTGGGCCATGGCTGAAGACAGTTCTCAAGTGCCACAT CCCAGATCCTTCTGAGTTCTTCTCCCAGCTGAGCTCCCAGCATGGGGGAG ACCTTCAGAAATGGCTCTCCTCGCCTGTCCCCTTGTCCTTCTTCAGCCCCA GTGGCCCTGCCCCTGAGATCTCTCCGCTGGAAGTGCTCGACGGAGATTCC AAGGCCGTGCAGCTGCTCCTGTTACAGAAGGACTCTGCCCCTTTACCCTC GCCCAGCGGCCACTCACAGGCCAGCTGCTTCACCAACCAGGGCTACTTCT TCTTCCATCTGCCCAATGCCTTGGAGATCGAATCCTGCCAGGTGTACTTC ACCTATGACCCCTGTGTGGAAGAGGAGGTGGAGGAGGATGGGTCAAGGC TGCCCGAGGGATCTCCCCACCCACCTCTGCTGCCTCTGGCTGGAGAACAG GATGACTACTGTGCCTTCCCGCCCAGGGATGACCTGCTGCTCTTCTCCCC GAGCCTCAGCACCCCCAACACTGCCTATGGGGGCAGCAGAGCCCCTGAA GAAAGATCTCCACTCTCCCTGCATGAGGGACTTCCCTCCCTAGCATCCCA TGACCTGATGGGCTTACAGCGCCCTCTGGAGCGGATGCCGGAAGGTGAT GGAGAGGGGCTGTCTGCCAATAGCTCTGGGGAGCAGGCCAGTGTCCCAG AAGGCAACCTTCATGGGCAAGATCAGGACAGAGGCCAGGGCCCCATCCTG ACCCTGAACACCGATGCCTATCTGTCTCTTCAAGAACTACAGGCCCAAGA TTCAGTCCACCTAATA

	TGF βR1	TGCCATAACCGCACTGTCATTCACCACCGTGTGCCAAATGAAGAGGATCC ATCACTAGATCGCCCTTTCATTTCAGAGGGCACCACCTTAAAAGATTTAA TTTATGATATGACAACATCAGGGTCTGGATCAGGTTTACCACTGCTTGTT CAAAGAACAATTGCCAGGACCATTGTGTTACAAGAAAGCATTGGCAAAGG TCGGTTTGGAGAAGTTTGGCGAGGCAAATGGCGGGGAGAAGAAGTTGCT GTGAAGATATTCTCTTCTAGAGAAGAGCGTTCATGGTTCCGAGAGGCAGA GATTTATCAGACTGTAATGTTACGCCATGAAAATATCCTGGGATTTATAG CAGCAGACAACAAAGACAATGGGACATGGACGCAGCTGTGGTTGGTGTC AGATTATCATGAGCATGGATCCCTTTTCGATTACTTGAATAGATACACTG TTACTGTGGAAGGAATGATCAAGCTTGCTCTGTCCACAGCAAGTGGTCTT GCCCATCTTCACATGGAGATTGTTGGTACCCAAGGAAAACCAGCTATTGC CCATAGAGATTTGAAATCAAAGAATATCTTGGTGAAGAAAAATGGAACCT GTTGTATTGCAGACTTGGGACTTGCTGTGAGACATGATTCTGCCACAGAT ACAATTGATATTGCTCCAAACCACAGAGTAGGCACTAAAAGGTACATGGC CCCTGAAGTTCTAGATGATTCCATAAATATGAAACATTTTGAATCCTTCAA ACGCGCTGACATCTATGCAATGGGCTTAGTGTTCTGGGAAATTGCTCGAC GCTGTTCTATTGGTGGAATCCATGAAGACTATCAGTTGCCTTATTATGAT CTTGTACCTTCTGATCCATCGGTTGAAGAAATGAGAAAAGTAGTTTGCGA ACAGAAGTTAAGGCCAAATATTCCAAACAGATGGCAGAGCTGTGAGGCCT TGAGAGTGATGGCTAAAATTATGAGAGAATGCTGGTATGCCAATGGAGCA GCAAGGCTGACAGCTTTGCGAATTAAAAAAACATTGTCACAACTCAGCCA ACAGGAAGGCATCAAAATG
	CD3 ζ	AGAGCAAAATTCAGCAGGAGTGCAGAGACTGCTGCCAACCTGCAGGACCC CAACCAGCTCTACAATGAGCTCAATCTAGGGCGAAGAGAGGAATATGACG TCTTGGAGAAGAAGCGGGCTCGGGATCCAGAGATGGGAGGCAAACAGCAG AGGAGGAGGAACCCCCAGGAAGGCGTATACAATGCACTGCAGAAAGACAA GATGGCAGAAGCCTACAGTGAGATCGGCACAAAAGGCGAGAGGCGGAGAG GCAAGGGGCACGATGGCCTTTACCAGGGTCTCAGCACTGCCACCAAGGAC ACCTATGATGCCCTGCATATGCAGACCCTGGCCCCTCGCTAA

Figure 12. Amino acid sequence of CAR construct.

		Amino acid sequence
Extracellular domain	Signal peptide	ATMASPLTRFLSLNLLLLGFSIILGSGEA
	V_H	QVQLKQSGAEFVKPGASVKISCKTSGYTFTDGYMNWVEQKPGQGLEWIGR IDPDSGDTRYNQKFQGKATLTRDKSSSTVYMDLRSLTSEDSAVYYCARAPY IADIGEAFDYWGQGTMVTVSS
	Linker	GGGGSGGGGSGGGG
	V_L	DIVLTQSPSSLAVSAGDKVTINCKSSQSLLSGGYNYLAWYQQKTGQSPKLLIY FTSTRHTGVPDRFIGSGSGTDFTLTINSFQTEDLGDYYCQHHYGTPLTFGDG TKLEIK
Transmembrane domain	CD28	DIVLTQSPSSLAVSAGDKVTINCKSSQSLLSGGYNYL
		AWYQQKTGQSPKLLIYFTSTRHTGVPD
		RFIGSGSGTDFTLTINSFQTEDLGDYYCQHHYGTPLTFGDGTKLEIK
Intracellular domain	IL2R β	GFFSCVYILVKCRYLGPWLKTVLKCHIPDPSEFFSQLSSQHGGDLQKWLSSPV PLSFFSPSGPAPEISPLEVLDGDSKAVQLLLLQKDSAPLPSPSGHSQASCFTNQ GYFFFHLPNALEIESCQVYFTYDPCVEEEVEEDGSRLPEGSPHPPLLPLAGEQ DDYCAFPPRDDLLLFSPSLSTPNTAYGGSRAPEERSPLSLHEGLPSLASRDLM GLQRPLERMPEGDGEGLSANSSGEQASVPEGNLHGQDQDRGQGPILTLNTD AYLSLQELQAQDSVHLI
	TGF β R1	CHNRTVIHHRVPNEEDPSLDRPFISEGTTLKDLIYDMTTSGSGSGLPLLVQRT IARTIVLQESIGKGRFGEVWRGKWRGEEVAVKIFSSREERSWFREAEIYQTV MLRHENILGFIAADNKDNGTWTQLWLVSDYHEHGSLFDYLNRYTVTVEGM IKLALSTASGLAHLHMEIVGTQGKPAIAHRDLKSKNILVKKNGTCCIADLGLA VRHDSATDTIDIAPNHRVGTKRYMAPEVLDDSINMKHFESFKRADIYAMGLV FWEIARRCSIGGIHEDYQLPYYDLVPSDPSVEEMRKVVCEQKLRPNIPNRWQ SCEALRVMAKIMRECWYANGAARLTALRIKKTLSQLSQQEGIKM
	CD3 ζ	RAKFSRSAETAANLQDPNQLYNELNLGRREEYDVLEKKRARDPEMGGKQQ RRRNPQEGVYNALQKDKMAEAYSEIGTKGERRRGKGHDGLYQGLSTATKD TYDALHMQTLAPR

Transfection and Transduction of CARs.

This CAR was transfected into HEK293FT cells by lipofection. The expression vector and packaging vectors were used to produce a lentiviral vector in HEK293FT cells. Harvested lentivirus has been tested in EL4 and HEK293FT cells, and the expression of ZsGreen fluorescence could be confirmed (Fig 13 A and B). I have confirmed that fluorescence increases as the number of virus supernatants increase.

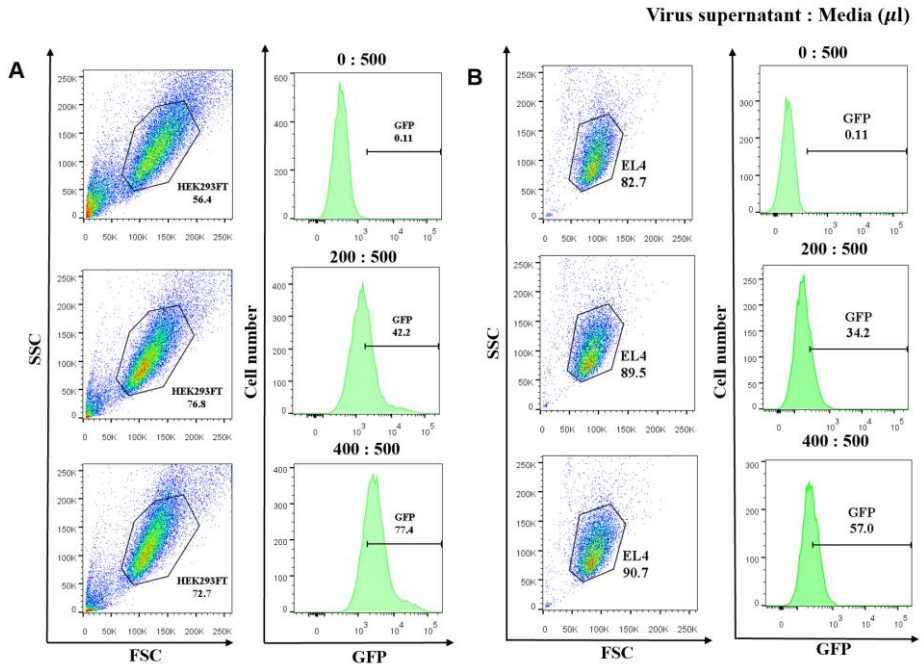

Figure 13. Transduction of CAR to cell line.

The expression of CAR was confirmed by ZsGreen1 fluorescence in pLVX-IRES-ZsGreen1 vector. The transduction of lentivirus with CAR was checked in HEK293FT cells (A) and EL4 cells (B). The virus supernatants were added according to quantities (0μl, 200μl and 400μl from the left side).

Production of a cell line expressing mouse CD40L.

I aimed to generate specific cell line expressing CD40L as antigen for CAR. DNA for CD40L was obtained from mouse $CD4^+$ cell. I generated CD40LF and CD40LR primer, which contained the part of 5' end of the CD40L gene, and the 3' end of the CD40L gene, respectively (Table 11). And, the primer sequence with specific restriction endonuclease site (F: Bgl II, R: EcoR I) is incorporated into the PCR product during amplification (Fig 14 A). This PCR product was extracted and cloned to the T vector. The transformation products were screened using a plate with antibiotics for selection. The colonies were confirmed by PCR at the expected DNA size. The pDNA can be cleaved with appropriate restriction endonuclease (Bgl II and EcoR I) (Fig 14 B). The cleavage reaction results in the production of the linearized strand of pmCherry-C1 vector and the *Cd40l* gene as insertions with the specific restriction endonuclease site at the end of pDNA, respectively. And this *Cd40l* gene to be cloned into the pmCherry-C1 vector by T4 ligase, and transformed. The transformation product was screened using a plate with antibiotics for selection. The colonies were identified by PCR at the estimated DNA.

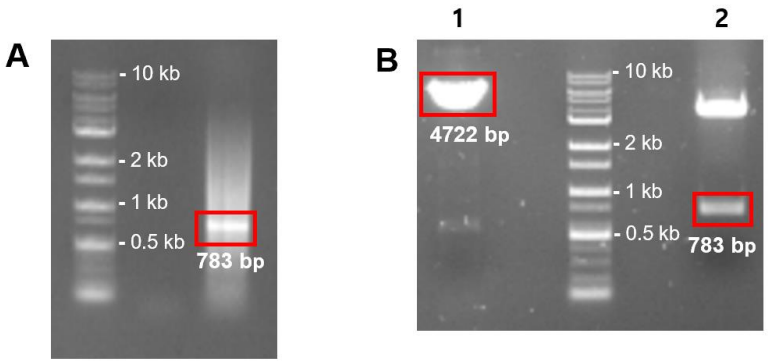

Figure 14. Generation of a pmCherry-C1 vector expressing CD40L.

A) A PCR fragment of 783 bp from mouse CD40L gene. The PCR was implemented with CD40LF/CD40LR primer sets and cDNA of mouse $CD4^+$ T cell as a template. B) Separation of the Bgl II and EcoR I digested pDNA. Lane 1 represents the linearized pmCherry-C1 vector. Lane 2 represents the separated pDNA of T vector including CD40L gene. The fragment estimated size 4722 bp (pmCherry-C1 vector) and 783 bp (CD40L) were extracted and purified.

CD40LF	**AGATCT** ATGATAGA AACATAC
CD40LR	**GAATTC** TCAGA GTTTGAGTAA G

Table 11. The oligonucleotide sequences for the acquisition of the CD40L gene.

The PCR product was amplificated with specific restriction enzyme site using these oligonucleotide primers. The bold lettering indicates restriction endonuclease sites.

This vector expressing CD40L were transfected into HEK293FT cells by lipofection. And the expression of mCherry fluorescence could be confirmed by FACS and fluorescence microscope (Fig15 A and B). A small amount of vector pDNA with CD40L was transfected. The mCherry fluorescence and CD40L expression by anti-CD40L mAb was confirmed.

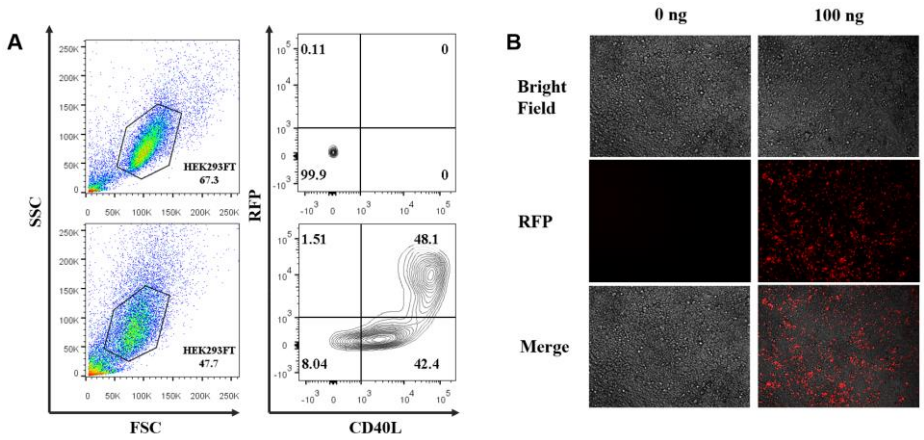

Figure 15. Transfection of a vector expressing CD40L gene.

A) The pmCherry-C1 vector with CD40L was transfected to HEK293FT cell in different quantity (0ng, 100ng from the top side). Representative plot of mCherry and CD40L expression of transfected HEK293FT cells. B) Expression of mCherry fluorescence in transfected HEK293FT cell with different quantity.

Activation of EL4 cell with CAR

I confirmed the expression of CD40L gene in HEK293FT Cell. So, I checked the activation of EL4 cell including CAR, using HEK293FT cell expressing CD40L. The response of transduced EL4 cell to the CD40L as antigen was examined by CD69 expression level. The anti-CD3/CD28 mAb molecule promoted the expression of CD69 in EL4 cells (Fig 16 B). The EL4 cells co-cultured with HEK293FT cells were also expressed the CD69 (Fig 16 C). And, Transduced EL4 cells co-cultured with transfected HEK293FT cells were expressed CD69 (Fig 16 D). But the expression level was higher in transfected HEK293FT cell co-culture, compared in not transfected HEK293FT cell.

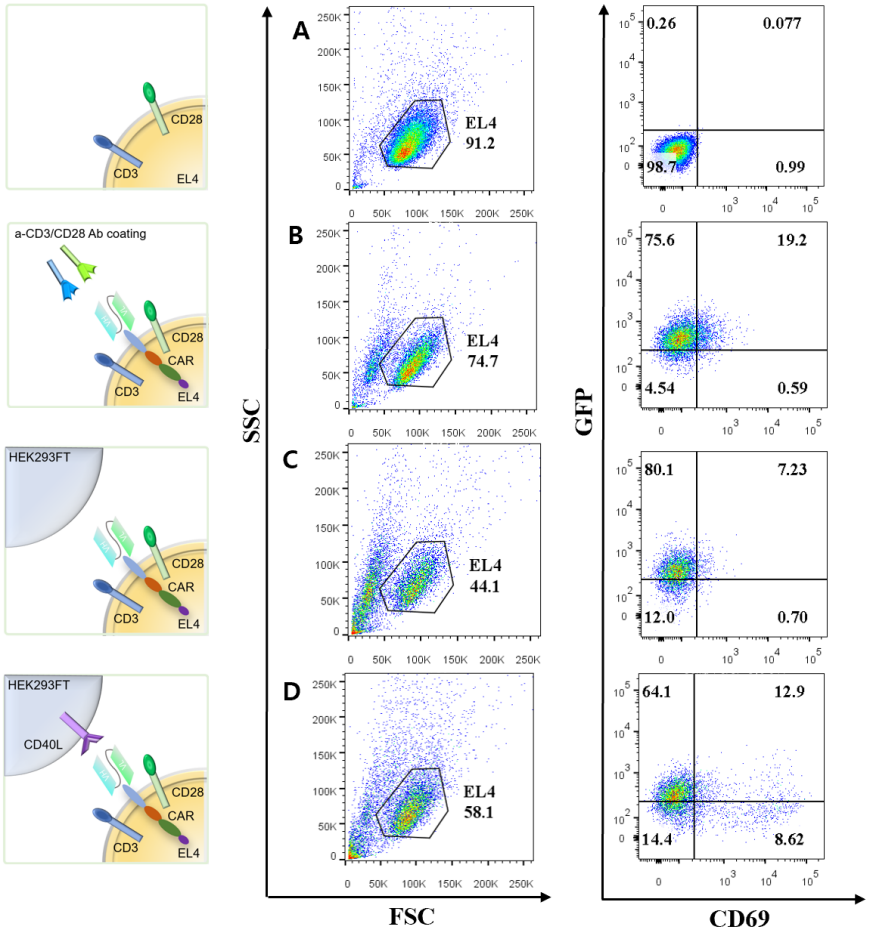

Figure 16. Co-culture effect of EL4 cell expressing CAR on the response of HEK293FT cell expressing CD40L.

The fluorescence expression level of EL4 cell (A). Transduced EL4 cell co-culture effect in (B) anti-CD3/CD28 mAb, (C) HEK293FT cells, and (D) transfected HEK293FT cells. Representative plot of GFP and CD69 in EL4 cells.

DISCUSSION

I describe a unique, useful strategy to generate CAR. This CAR has CD40L specific scFv, an extracellular spacer comprised of mouse CD28 hinge, CD28 transmembrane domain, CD28 costimulatory domain, the intracellular domain of IL-2Rβ and TGF-βR1, and an intracellular CD3ζ signaling region. This new CAR design is unusual in that it has the identical costimulatory and CD3ζ signaling regions but also include the intracellular domain of IL-2Rβ and TGF-βR1 compared to conventional CARs. The inclusion of the intracellular domain of IL2-Rβ and TGF-βR1 enabled conversion of naïve T cells to iTreg with CARs in an antigen-dependent manner. In response to an antigen, I expect this CAR construct converts the naïve T cell to Treg, and then this converted t cell could suppression function and block the effector T cell by binding these cell directly to CD40L.

Here, I generated a CAR construct using cloning and PCR techniques. I acquired each gene by PCR using taq and pfu polymerase for preventing mutation. At this time, considering the site to be ligated, a restriction endonuclease site was inserted in the primer. The restriction site also constituted a condition that did not truncate each gene. The genes from PCR were extracted, purified, and transformed into T vector. The genes obtained in the form of pDNA were cloned again for ligation, or extended by OE-PCR technique. And finally, the CAR construct was designated.

The CAR was transfected into HEK293FT cells by lipofection. And the harvested virus was transduced in HEK293FT cell for test and EL4 cell. The

expression of ZsGreen1 fluorescence was detected by FACS. The percentage of the transduced cell with CAR construct was higher in HEK293FT cell than EL4 cell. But, I demonstrated that these CARs could be efficiently delivered into the mouse T cell line by lentiviral transduction.

Moreover, I generated the HEK293FT cell expressing CD40L as the antigen of CAR. The CD40L gene was derived from mouse $CD4^+$ T cell. And, this gene was cloned into a pmCherry-C1 vector. And I confirmed the expression of mCherry fluorescence by FACS and fluorescence microscopy. The expression level was 40% in the amount of 100 ng pDNA with CD40L gene. I not shown here, I confirmed that the expression level was increased at a larger amount of pDNA. Furthermore, I checked the activation of EL4 cells including CAR construct, using HEK293FT cells expressing CD40L. The response of transduced EL4 cell to the CD40L as the antigen of the CAR was checked by CD69 expression level. And, the CD69 expression level was higher in transfected HEK293FT cells co-culture set compared in not transfected HEK293FT cells.

The CARs have principally been used to generate cancer-specific effector T cells. And this CAR-T therapy had a positive effect and resulted in the development of new cell therapy (27). Furthermore, CAR-T therapy was evolved in Treg. Many studies of CAR-Treg have been performed, and its efficacy has been proved. The prevention efficacy of CAR-Treg with MHC specificity was demonstrated in allograft rejection. Also, The efficacy of human CAR Tregs was demonstrated in the prevention of xeno-GVHD (28). These

CAR-Treg designs suggest a successive useful possibility to induce graft-specific tolerance as a new therapy.

In the transplantation model, the finding of the new antigen of the donor offers an essential role. Also, the CAR design has higher specificity result in off-target effect without cross-reactivity to avoid an unexpected effect (29). In current trails, the Tregs for adoptive therapy was used to treat patients (30). But there is a limit to long expansion time, which is critical severely ill patients. However, the A2-CAR Tregs have engineered with 'off-the-shelf' strategy with significant functional effect and short expansion time results in powerful expected function for treatment (31).

I generated CAR-T cells which can convert into CAR-iTreg upon antigen simulation, and I accordingly expected this CAR-T cells to be a therapeutic tool to effectively regulate the immune response in transplantation or autoimmune disease model. This CAR with CD40L specificity might offer a novel strategy to achieve tolerance after organ transplantation (32). And also, based on CAR construct explained here, the CAR specific for different new antigens might provide the significant potential of the CAR technology. This work I described here is the first proof-of-concept for the generation of CAR-iTreg with potential to be used therapeutically.

REFERENCES

1) Boyiadzis MM, Dhodapkar MV, Brentjens RJ, Kochenderfer JN, Neelapu SS, Maus MV, et al. Chimeric antigen receptor (CAR) T therapies for the treatment of hematologic malignancies: clinical perspective and significance. Journal for immunotherapy of cancer. 2018;6(1):137.

2) Sadelain M, Brentjens R, Rivière I. The basic principles of chimeric antigen receptor design. Cancer discovery. 2013;3(4):388-98.

3) Zhang C, Liu J, Zhong JF, Zhang X. Engineering CAR-T cells. Biomarker research. 2017;5(1):22.

4) Linsley PS, Ledbetter JA. The role of the CD28 receptor during T cell responses to antigen. Annual review of immunology. 1993;11(1):191-212.

5) June CH, Sadelain M. Chimeric antigen receptor therapy. New England Journal of Medicine. 2018;379(1):64-73.

6) Budde LE, Zaia JA. CD19 CAR-T therapy and sepsis: dancing with the devil. Blood. 2018;131(1):7-8.

7) Lim WA, June CH. The principles of engineering immune cells to treat cancer. Cell. 2017;168(4):724-40.

8) Boardman D, Maher J, Lechler R, Smyth L, Lombardi G. Antigen-specificity using chimeric antigen receptors: the future of regulatory T-cell therapy? Biochemical Society Transactions. 2016;44(2):342-8.

9) Sharabi A, Tsokos MG, Ding Y, Malek TR, Klatzmann D, Tsokos GC. Regulatory T cells in the treatment of disease. Nature Reviews Drug Discovery. 2018.

10) Josefowicz SZ, Lu L-F, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. Annual review of immunology. 2012;30:531-64.

11) Kim YC, Zhang A-H, Su Y, Rieder SA, Rossi RJ, Ettinger RA, et al. Engineered antigen-specific human regulatory T cells: immunosuppression of FVIII-specific T-and B-cell responses. Blood. 2015;125(7):1107-15.

12) Noyan F, Zimmermann K, Hardtke-Wolenski M, Knoefel A, Schulde E, Geffers R, et al. Prevention of allograft rejection by use of regulatory T cells with an MHC-specific chimeric antigen receptor. American Journal of Transplantation. 2017;17(4):917-30.

13) Boardman DA, Philippeos C, Fruhwirth GO, Ibrahim MA, Hannen RF, Cooper D, et al. Expression of a chimeric antigen receptor specific for donor HLA class I enhances the potency of human regulatory T cells in preventing human skin transplant rejection. American Journal of Transplantation. 2017;17(4):931-43.

14) Chen W, Jin W, Hardegen N, Lei K-j, Li L, Marinos N, et al. Conversion of peripheral CD4+ CD25− naive T cells to CD4+ CD25+ regulatory T cells by TGF-β induction of transcription factor Foxp3. Journal of Experimental Medicine. 2003;198(12):1875-86.

15) Weiss A, Attisano L. The TGFbeta superfamily signaling pathway. Wiley Interdisciplinary Reviews: Developmental Biology. 2013;2(1):47-63.

16) Zheng SG, Wang J, Horwitz DA. Cutting edge: Foxp3+ CD4+ CD25+ regulatory T cells induced by IL-2 and TGF-β are resistant to Th17 conversion by IL-6. The Journal of Immunology. 2008;180(11):7112-6.

17) Ross SH, Cantrell DA. Signaling and function of interleukin-2 in T

lymphocytes. Annual review of immunology. 2018;36:411-33.

18) Ara A, Ahmed KA, Xiang J. Multiple effects of CD40–CD40L axis in immunity against infection and cancer. ImmunoTargets and therapy. 2018;7:55.

19) Lanschuetzer CM, Olasz EB, Lazarova Z, Yancey KB. Transient anti-CD40L co-stimulation blockade prevents immune responses against human bullous pemphigoid antigen 2: implications for gene therapy. Journal of Investigative Dermatology. 2009;129(5):1203-7.

20) Robles-Carrillo L, Meyer T, Hatfield M, Desai H, Dávila M, Langer F, et al. Anti-CD40L immune complexes potently activate platelets in vitro and cause thrombosis in FCGR2A transgenic mice. The Journal of Immunology. 2010;185(3):1577-83.

21) Shock A, Burkly L, Wakefield I, Peters C, Garber E, Ferrant J, et al. CDP7657, an anti-CD40L antibody lacking an Fc domain, inhibits CD40L-dependent immune responses without thrombotic complications: an in vivo study. Arthritis research & therapy. 2015;17(1):234.

22) Daley S, Cobbold SP, Waldmann H. Fc-disabled anti-mouse CD40L antibodies retain efficacy in promoting transplantation tolerance. American Journal of Transplantation. 2008;8(11):2265-71.

23) Guedan S, Calderon H, Posey Jr AD, Maus MV. Engineering and design of chimeric antigen receptors. Molecular Therapy-Methods & Clinical Development. 2018.

24) Gilliland L, Norris N, Marquardt H, Tsu T, Hayden M, Neubauer M, et al. Rapid and reliable cloning of antibody variable regions and generation of recombinant single chain antibody fragments. Tissue antigens. 1996;47(1):1-

20.

25) Wu C-Y, Roybal KT, Puchner EM, Onuffer J, Lim WA. Remote control of therapeutic T cells through a small molecule–gated chimeric receptor. Science. 2015;350(6258):aab4077.

26) Krause A, Guo H-F, Latouche J-B, Tan C, Cheung N-KV, Sadelain M. Antigen-dependent CD28 signaling selectively enhances survival and proliferation in genetically modified activated human primary T lymphocytes. Journal of Experimental Medicine. 1998;188(4):619-26.

27) Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor–modified T cells in chronic lymphoid leukemia. New England Journal of Medicine. 2011;365(8):725-33.

28) MacDonald KG, Hoeppli RE, Huang Q, Gillies J, Luciani DS, Orban PC, et al. Alloantigen-specific regulatory T cells generated with a chimeric antigen receptor. The Journal of clinical investigation. 2016;126(4):1413-24.

29) Drent E, Themeli M, Poels R, de Jong-Korlaar R, Yuan H, de Bruijn J, et al. A rational strategy for reducing on-target off-tumor effects of CD38-chimeric antigen receptors by affinity optimization. Molecular Therapy. 2017;25(8):1946-58.

30) Dall'Era M, Pauli ML, Remedios K, Taravati K, Sandova PM, Putnam AL, et al. Adoptive Treg cell therapy in a patient with systemic lupus erythematosus. Arthritis & Rheumatology. 2019;71(3):431-40.

31) Bluestone JA, Buckner JH, Fitch M, Gitelman SE, Gupta S, Hellerstein MK, et al. Type 1 diabetes immunotherapy using polyclonal regulatory T cells. Science translational medicine. 2015;7(315):315ra189-315ra189.

32) Elgueta R, Benson MJ, De Vries VC, Wasiuk A, Guo Y, Noelle RJ. Molecular mechanism and function of CD40/CD40L engagement in the immune system. Immunological reviews. 2009;229(1):152-72.

국문 초록

서론: 조절 T 세포는 흉선에서 유래된 억제 능력을 가진 세포로 자가항원에 대한 면역관용을 유지하는데 중요한 역할을 한다. 본 연구에서는 키메라 항원 수용체를 사용하여 CD40L 자극을 받으면 미감작 T 세포를 조절 T 세포로 분화시키는 새로운 기술을 개발하였다. 키메라 항원 수용체는 세포 외 영역은 주로 항체에서 유래되고, 세포 내 신호전달 영역은 T 세포의 신호전달 부분에서 유래된 합성 단백질이다. 키메라 항원 수용체의 세포내 영역에 TGF-β 수용체의 세포질 부분과 IL-2Rβ의 신호전달 영역 부분을 포함시켰다. 그 결과, 2 세대 키메라 항원 수용체를 기반으로 하여, MR1 단일 항체의 scFv와 CD28, IL-2Rβ, TGF-βR1, CD3ζ의 부분을 클로닝을 통해 합성하였고 CD40L 의 특이성을 가지는 키메라 항원 수용체를 제작하였다.

방법: 마우스 $CD4^+$ T 세포는 MACS 시스템을 이용하여 분리하였다. 그리고 이 유전자들은 키메라 항원 수용체를 구성하는데 사용되었다. 각 유전자들은 중합효소연쇄반응에 의해 특정 올리고뉴클레오티드를 사용하여 증폭하고 연결시켜 재조합 유전자를 합성하였다. 합성된 키메라 항원 수용체는 렌티바이러스 벡터에 삽입되었다. 이 벡터와 운반벡터들을 사용하여, 키메라 항원 수용체를 암호화하는

DNA 를 포함하는 재조합 렌티바이러스를 만들었으며, 수확된 바이러스는 EL4 세포에 형질전환에 사용되었다.

결과: 키메라 항원 수용체 구조는 마우스 cDNA 로부터 얻은 후 합성하였다. 완성된 키메라 항원 수용체는 렌티바이러스 벡터에 삽입되었다. 그리고 키메라 항원수용체의 발현은 pLVX-IRES-ZsGreen1 벡터에 있는 ZsGreen 형광으로 확인하였다. 또한 키메라 항원 수용체에 특이적으로 결합하는 CD40L 를 발현하는 세포주를 만들었고 mCherry 형광을 이용하여 발현을 확인하였다. 키메라 항원수용체를 발현하는 EL4 세포와 CD40L 를 발현하는 HEK293FT 세포를 함께 배양하고, 키메라 항원수용체를 발현하는 EL4 세포의 활성여부를 CD69 의 발현을 통해 확인하였다.

결론: 본 연구에서는 새로운 키메라 항원 수용체를 발현시킬 수 있는 재조합 유전자를 합성하였는데, 기존의 수용체와 비교하였을 때, IL-2Rβ와 TGF-βR1 의 세포내 영역을 포함한다는 점에서 특별하다. IL-2Rβ와 TGF-βR1 의 세포내 영역은 항원 의존적으로 미감작 T 세포가 조절 T 세포로 전환되는 것을 가능하게 한다. 이러한 키메라 항원수용체 구조가 이식 모델과 자가면역질환 모델에서 사용된다면, 유의미한 면역억제능력을 유도할 수 있을 것으로 기대한다. CD40L

특이성을 가지는 키메라 항원 수용체는 장기 이식후에 면역관용을 유도할 수 있는 새로운 전략을 제공할 수도 있을 것이다.

주요어 : 조절 T 세포, 키메라 항원 수용체, 중합효소 연쇄반응, 클로닝, 형질 전환, 형질도입

학 번 : 2017-22413